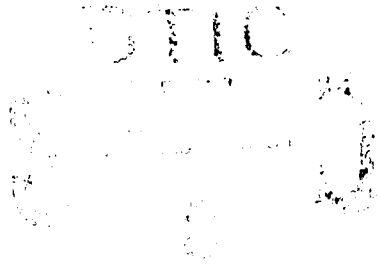

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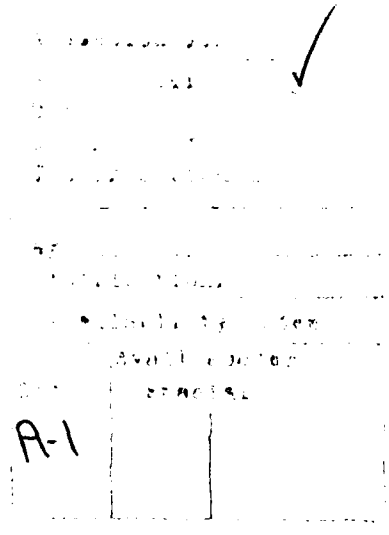

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The past three years has seen considerable advancements in our understanding of the fundamental basis of laser interaction with retinal and ocular tissue. In this section we will review our achievements during the past three years of support by the Ocular Hazards Program of the United States Letterman Army Institute of Research. This support has resulted in 15 published papers (see references 1-9 and 14-19 in the Bibliography). These papers have reported for the first time second harmonic generation in rhodopsin. The data provide an understanding of the very efficient second harmonic generation that is occuring in visual pigments. The fundamental understanding that we have obtained begins to elucidate the interactions that could underly the psychophysical phenomena that have been indicated green sensation with Nd:YAG laser illumination. In addition, these results are leading to a more detailed understanding of the photodynamics of the rhodopsin spectral alterations under both one and two photon illumination. This photodynamics is the basic information that is required in order to design the constraints that must be met in order to develop photodynamic filters. Furthermore, our results have shown that when a laser is absorbed by the retinal chromophore of the visual pigment not only is the retinal altered but also the lysine that is covalently attached to the retinal is also affected by interaction with laser light. Finally, we have shown that specific laser irradiation induces free radical production in ocular tissue and this has the ability to cause significant damage to the affected ocular tissue. In the sections below we will elaborate on each of these advances and we will also describe the continuing exciting developments in near-field microscopy that are aimed at providing super-resolution light microscopic images of dynamic alterations in retinal tissue with laser irradiation.

Rhodopsin Second Harmonic Generation

Understanding the fundamental mechanisms of photon excitation in rhodopsin is of crucial importance in elucidating the dynamic changes in the visual system that fix the characteristics of the dynamic protection systems that have to be developed. An understanding of these fundamental mechanisms of photon excitation is essential not only in terms of the practical problems of protection and the psychophysical response to such commonly used hardware as Nd:YAG laser range finders, but, also in terms of elucidating one of the central processes in

biophysics that remains unresolved inspite of decades of investigations in many laboratories throughout the world.

The first step in the laser excitation process is the absorption of a photon by the retinal chromophore that is found in all visual pigments. The absorption process induces electronic and structural alterations in the retinal. These alterations are central to the interactions that underly the dynamic changes and the function of all retinal pigments. The decades of linear spectroscopic investigations on such proteins have not clarified the light induced electron redistribution in rhodopsin that controls all the dynamic alterations in these pigments.

During the past three years our group has pioneered the use of the non-linear optical technique of second harmonic generation as a probe of the light induced dynamic alterations in the retinal chromophore [1-8] and in order to understand the various wavelengths that are produced when a variety of pulsed and cw lasers enter the eye. In this technique a laser beam illuminates membrane bound rhodopsin and this interaction produces a beam of light at a frequency that is twice that of the illuminating (fundamental) laser beam. The intensity of this short wavelength (second harmonic) light is dependent on the square of the intensity of the fundamental beam and on the the second order term that describes the polarizability of the electrons in the molecule. This second order polarizability is related to the induced dipole of the charge transfer associated with the main optical absorption. The second order perturbation theory that defines the relationship of the second order polarizability to the induced dipole includes an energy denominator that allows for either the fundamental or the second harmonic beam to enhance the signal due to resonance of one or both of these beams with the principal electronic transition. Therefore, it is possible to shine an infrared fundamental beam on the sample and produce visible second harmonic light that is in resonance with (close to) the visible absorption of the retinylidene chromophore. This enhances the signal of the chromophore above the background of the second harmonic generation that could originate from all the other groups in the protein. The important aspect of such an experiment for retinylidene proteins is the simplifications that result in the interpretation of the second harmonic signals if the molecule being investigated has a long molecular

axis. In this case a third rank tensor which in Cartesian coordinates has 27 tensor elements reduces to simply two independent non-vanishing components. From these two components can be calculated both the angle of the chromophore relative to the normal to the plane of the film containing the chromophores and the second order polarizability that is related to the induced dipole. Thus, in terms of theoretical, experimental and the practical considerations of understanding the light that is produced when lasers of various wavelengths enter the eye it is important to investigate second second harmonic generation of retinylidene proteins.

The results reported in the eight papers published to date on this grant [1-8] can be succinctly summarized. The measurements clearly showed that rhodopsin is one of the largest generators of second harmonic light that has been discovered. An understanding of this large generation of second harmonic photons is important because it means that laser light entering the eye can also effectively produce other wavelengths. The second harmonic signal also provides a wealth of other information. For example, the data for the first time was capable of sensing the absolute direction of the retinal chromophore in the membrane. The data have shown that for membrane bound rhodopsin, which has not been subjected to any pre-radiation with a laser, the angle of the chromophore is 65^0. Such unirradiated chromophores have extremely large light-induced dipole moments which correspond to an alteration of 24 Debyes as a result of light excitation. To understand further the dynamics of these interactions that lead to the large non-linear response of rhodopsin we developed the technique of time-delayed second harmonic generation and followed the dynamics of the changes in the second harmonic signal which corresponded to electronic and structural relaxation after light excitation. We then went on to investigate alterations in these parameters with pre-irradiation protocols. One of the most interesting results was the lowering of the induced dipole of the chromophore from 24 Debyes to 12 Debyes after the retina was exposed to low intensity ($1 mW/mm^2$) helium neon laser illumination for 1 second. Detailed histological investigations were performed in the Hadassah Hospital Ocular Pathology laboratory and no morphological alterations could be detected with this irradiation protocol. This was in agreement with numerous previous investigations. In addition, the alteration observed in the induced dipole was paralleled with a reduction of the angle of the chromophore angle from 65^0 to 50^0.

Furthermore, as will be indicated in the next section this data was in agreement with alterations seen in the Fourier transform infrared measurements to be described in the next section.

In summary, over the past ten years second harmonic generation has been very effectively developed in terms of its application to a variety of physical problems. In the recent past chemists have been active in this area as a source of new molecules which exhibit high non-linearities. These investigations have been mainly aimed at developing new non-linear devices for practical application. In spite of this activity in both physics and chemistry there has been a paucity of investigations in which the technique had been used to answer specific questions on a biological system. Thus our investigations over the past three years have stood out as the first efforts to understand both the non-linear interaction of light with a biological system and to extract from these measurements a better understanding of the visual system and practical methods to protect this system against damage.

The First Visualization of a Crucial Lysine in All Visual Pigments

Rhodopsin sits in the disk membrane which is formed of a lipid bilayer. Embedded in the rhodopsin protein is the light absorber of the visual system which is retinal. Retinal is covalently attached in all visual pigments by a crucial lysine amino acid residue. Although there have been decades of work on the retinal chromophore there is no information on what happens to this crucial lysine that is covalently bound to the retinal when light interacts with rhodopsin.

When light is absorbed by all rhodopsins they undergo a series of color changes. Within 750 femtoseconds of photon absorption there is a 40 nm red-shift in the rhodopsin visible spectrum and this occurs in all rhodopsins whether the visual pigment comes from a cone or a rod, or whether it comes, as was discovered about 20 years ago, from a bacterium. As a result of this initial photon event there is a series of thermal events which stimulate similar alterations in the biochemistry of both rod and cone cells.

To show that this first fundamental light event takes place in a few

hundred femtoseconds we have completed absorption measurements at 520nm and 570nm with a colliding pulse mode dye laser having a pulse width of 160fsec. From these measurements (see Figure 1) we can detect in the green at 520nm a bleaching of the initial pigment state after light excitation and in concert with this bleaching is an increase in absorption in the yellow at 570nm corresponding to the production of the photochemical product. We can study this first event non-kinetically even though it occurs in femtoseconds by freezing the state. In other words the photochemical product is a ground state and not an excited state and thus it can be trapped indefinitely by freezing.

With the ability to trap this important dynamic intermediate we can apply the sensitive technique of Fourier transform infrared spectroscopy (FTIR) to elucidate structural changes in both the retinal and the surrounding protein. The important advance that FTIR spectroscopy provides is that unlike normal monochrometer based spectroscopies which use a slit and prevent light from effectively getting from the sample into the monochromator to be analyzed, an interferometer, allows all of the light from the sample to be sent through a very big aperture for analysis. One of the significant aspects of this device is that it accepts much more signals than a normal spectral device and furthermore, a laser is used which gives an absolute calibration of each of the wavelengths so that exceptional difference spectra can be obtained. Thus the effect of laser radiation on a sample can be sensitively monitored with the difference spectra capabilities of FTIR.

With this introduction to the difference spectra capabilities of FTIR we focus on the investigation of the initial event in the dynamic absorption changes in vision. We are studying in these measurements [9] the first event which occurs in a few hundred femtoseconds. The alterations observed are seen in Figure 2. In Figure 2A is the difference FTIR spectrum between the initial pigment state (R) and the red state (B) in native rhodopsin. To obtain these spectra the pigment is placed inside the spectrometer and a spectrum is taken. Subsequently, light is shone on the sample and another spectrum is obtained. A difference spectrum is then generated and the changes induced by the light event in the pigment are recorded. The difference spectrum displayed in Figure 2B were recorded for rhodopsin with all the protons of lysine deuterated. To

obtain bovine rhodopsin with fully deuterated lysines bovine retina were maintained in cell culture for four days using an already established procedure [10]. The only difference from the published procedure is that the defined medium uses lysine synthesized with deuterons replacing all of the lysine protons. In the difference spectrum (Figure 2B) in which all of the lysines had their protons replaced with deuterons there were many changes. In fact all of the bands labelled in Figure 2A and B correspond to differeces due to lysine enrichment. Further experiments with lysine methylated at all positions except the active site [11] indicated that all the effects seen in these spectra are due to the active lysine. Thus by comparing these spectra we are readily able to differentiate for the first time between the bands that are due to the retinal and those due to the lysine covalently bound to the retinal. Until this sequence of experiments, this lysine, which is so crucial in visual pigments, was totally silent spectroscopically. So with this experiment we are able to differentiate the two major components that are involved in the light event. The retinal, the light absorbing species, and the covalently linked lysine which our results indicate is undergoing significant alteration.

With this information on the lysine vibrational modes we moved on to monitor possible alterations in these modes as a function of the low intensity laser irradiation that caused changes in the second harmonic signal. For these measurements the reader's attention is drawn to the intense peak in Figure 2A that lies between the 941 and 974cm^{-1} bands. This intense peak is due to retinal while the 941cm^{-1} band is due to lysine. This can clearly be seen in Figure 2B. In this Figure we see that lysine substitution complete abolishes the 941cm^{-1} peak but does not effect the intense band next to it. With this definitive assignment we monitor these two peaks in bovine rhodopsin obtained from a bovine retina which had been exposed to a 1mW/mm^2 of helium neon irradiation. We notice in these spectra several changes (see Figure 3) including a 3cm^{-1} alteration in the 941cm^{-1} band. However notice that the neighboring band that we have assigned to retinal remains unchanged. Other protein bands marked with a dot in Figure 3 are also seen to change. Thus, once again our data indicate that subtle structural changes are being detected with laser illumination protocols and these laser irradiation protocols show no gross morphological effects.

In summary this sequence of experiments completed with the support of the U. S. Army Ocular Hazards Program has developed and demonstrated subtle structural changes with low intensity laser irradiation using a new and exquisitely sensitive structural tool. It will be important to couple these findings with a continued sequence of measurements on various laser irradiation protocols in order to see the transition from low level laser effects to effects in which gross morphological changes can be detected.

Free Radical Formation with Laser Irradiation

We have also made significant advances during this period in terms of understanding the fundamental steps in laser induced ocular damage that is mediated by factors other than other than rhodopsin mediated effects. Specifically, we have applied an exciting new method to discover the presence of free radicals after laser irradiation of retinal tissue. The essence of the technique is the addition of salicylic acid to the tissue that is to be interrogated. If free radicals are produced in the aqueous medium by the laser radiation the addition of a hydroxyl group from the solvent to the salicylic acid takes place and this produces 2,3 dihydroxybenzoic acid (DHBA) and 2,5 DHBA. These products are then separated from the tissue using high performance liquid chromatography. The results from measurements that investigated the concentration of 2,3 DHBA and 2,5 DHBA with increasing energy densities of a Nd:YAG laser with a 10nsec pulse width on bovine retinal tissue are seen in Figure 4. In this Figure we see a definite increase in the 2,3 DHBA analog as a function of energy density of the Nd:YAG laser pulse. These measurements will continue on several types of ocular tissue and several different laser irradiation protocols will be used in our future efforts in this area. The data give new insight into some of the other biochemical processes that could cause significant damage to retinal tissue and could provide the initial switch for many of the drastic morphological changes that are detected with laser irradiation.

Light Microscopy to the Limits of Single Molecule Resolution

A decade of research, initiated with funds from the occular hazards

program, continues to come ever closer to the goal of utilizing the non-destructive capabilities of fluorescence light microscopy at resolutions that approach those that are more commonly associated with electron microscopy. Obviously, the microscope has been an instrument of great utility in cell biology and morphology. In general the application of microscopy in cell biology can be divided into two distinct areas. First, relatively low resolution images obtained with light microscopy that have given the cell biologist information on a whole variety of characteristics of cells from dynamics to the three dimensional characteristics of cells. Second, the very high resolution images that can be obtained with the electron microscope which have given the biologist a variety of new views on molecular structure and interaction as related to problems in cell biology. Even though such images of molecular aggregates are in high vacuum environments, which of course are not natural for the cellular system, there is still a great deal to be learned from these high resolution images obtained in non-ambient condition. For example, detailed information can be obtained on the organization of proteins in cell membranes or the structure of cellular organelles and it has even been possible to resolve the distribution of amino acids in the membrane for a crystalline protein such as bacteriorhodopsin. The ultimate in microscopy of cellular systems would be to combine the high resolution of the electron microscope with the capability of light microscopy to investigate cellular systems in dynamic, living environments. This is the goal of our continuing efforts in this direction. In essence we want to dynamically and without artifact view the molecular rearrangements that ensue after laser irradiation and although we are not there yet we believe that we are getting ever closer to this goal. The achievements of the past three years are summarized in this section.

Specifically this section deals with a new approach in light microscopy that attempts to achieve what seemed unthinkable a few years ago. The essence of this new approach is to realize that one of the najor constrains on the light microscope results from the fundamental characteristics of lens based imaging. These fundamental constraints arise fom the fact that lenses are limited in their resolution to approximately a quarter of the wavelength of the light that is being used for the imaging. Thus, for visible light of 500 nm the best theoretical resolution that can be achieved is around 150 nm. In practice, the best

resolution that has been achieved after deconvolving the aberrations found in the objective lens is ~0.2 microns. Our approach has been to replace the lens by a hole that is considerably smaller than the wavelength of light. We replace the lens with great trepidation because a lens is a parallel imaging device and, as will be seen, the hole that we use images in serial. Therefore by replacing the lens with a hole we have in fact considerably lengthened the time needed to obtain an image.

Now, how can a hole replace a lens to form an image? In principle, if a hole is brought very close to a surface then a resolution of approximately the diameter of the hole can be achieved. How near the surface does such a hole have to be brought? The answer depends on the resolution that has to be achieved. In general the hole/sample separation has to be approximately the diameter of the aperture size chosen. In essence, this means that for a resolution of 50nm, a hole of 50nm has to be chosen and one has to maintain a separation that is approximately 50nm. This distance over which the light wave does not spread out and where resolutions can be achieved that are related to the aperture diameter is called the near-field.

We have been developing optical instrumentation that use this important characteristic of the near-field in order to obtain resolutions far beyond what is achievable by all the lens-based, far-field instruments that exist. To achieve such optical imaging, two important characteristics must be obtained. The first is that a method must be found that allows the relatively cheap production of holes that can be relatively the best during the scanning procedure over the interest. To addition to this important achievement it is also required to attain stability and feedback that allows the hole surface distance to be maintained during the entire image formation process. The recent research on this area has been focused on solutions for both these problems. For the first problem there have been a number of interesting solutions. These colutions resulted in three distinct types of apertures that could be used for the imaging process. The first is simply a piece of glass that can be etched to a very sharp point. The type of structures used for such a hole at the point are either single crystals of quartz or optical fibers that can be etched to a point. The second is the formation of apertures in metal plates either by standard electron beam

lithography technology, that is widely applied in microelectronics, or by the use of small latex spheres that can be coated with a metal after they have been deposited on glass and then are removed by a sonication process. Finally, the solution used in our laboratory has been an extension of biological micropipettes which can be pulled diameters as small as 7.5nm.

The stability problems have been solved based upon the dramatic developments in all forms of scanned tip microscopy [12] that allow the positioning of a tip relative to a surface with great precision. The essence these technologies demand that small rigid structures have to be generated in which the tip and the sample are held together rigidly. This has been achieved through the variety of ingenious methodologies most of them based upon the use of a hollow cylinder piezuelectric ceramic that permits three-dimensional movement of the tip or in our case, an aperture for light with extremely high resolution and with a view that can be extended to as much as 0.1 mm.

In spite of these achievements a major problem that still has to be resolved for biological application of high resolution near-field scanning optical microscopy to cellular systems, is the problem of control of aperture/surface separation. There is no doubt that this has been one of the major strumbling blocks in the application of NSOM to biology. It has been a constant concern in our laboratory and although a final resolution has still not been achieved I have been never more confident of its resolution. One of the most promising avenues of investigation is the use of an observation well-known in cell biology. This is the measurement of changes in conductance as a micropipette approaches a surface of a cell. This has been used in methods such as patch clamping, to inform the researcher when the micropipette is close to the surface. It is quite possible that this will be a relatively simple solution to the problem of distance regulations in near-field microscopy as applied to cell biology. This is currently under active investigation in our laboratory.

The components that are listed above can be integrated into a near-field microscope in a simple fasion as is seen in Fig. 5. The microscope seen in this figure was built with little effort to accommodate biological problems. Its objective was to demonstrate the

essential aspects of near-field microscopy. As can be seen in the figure, the microscope sits on a series of metal plates that are separted by rubber tubing to allow for vibration isolation. The microscope is composed of a rough stage which is simply built out of motorized micrometers, and this rough stage leaves a sample which is sitting vertical to the vibrational isolation and is in close proximity to a cylindrical tube in which the pipette sits. The cylindrical tube allows for the X, Y and Z fine positioning of the pipette over the sample. The light that comes through the pipette and passes through the sample is focused onto a detector which sits behind the sample. Alternately, the sample can be bathed with light and the pipette could collect the light at localized points and could be directed to a photomultiplier. In both cases the sample is viewed in transmission microscopy.

Using this microscope, an interesting series of investigations have been performed on scanning in pipettes over holes of predetermined size. The results from one such study are seen in Fig. 6, in which the pipette is brought in steps closer to the aperture containing surface and images of the holes are produced. When the pipette is in the far-field, interferences can occur between the 514:5 nm light wave that emanates from the pipette and reflects from the surface. These interferences indicate that the pipette is not in the near-field. As the pipette approaches the surface, the intereference disappeares indicating that we are indeed in the near-field. When a pipette is within a distance from the surface that is approximately the diameter of the hole at the pipette tip, the image of the aperture in the metal plate is that of an intense beam of light and that is seen in Fig. 6. These are some of the best images obtained to date with NSOM. Several interesting observations can be made about these images. First, the data was obtained with a simple uncooled photomultiplier with a high dark count and the data was recorded with a lock-in system. Because of this primitive method of detection each scan took 15 mins. Nonetheless the signal to noise was excellent, greater than 200:1. This implies either that there is mechanical stability in the system that is much better than is required by the requirements of near-field microscopy or that the requirements of near-field microscopy are somewhat less drastic from the 30Å that has been quoted [13]. This question is currently being addressed by repeating the above measurements with very accurate distance regulation. This should

finally result in experimental information on the function that describes the spread of the radiation in the near-field.

In spite of the considerable success with this first generation of near-field microscopes [14-18], significant improvements have to be made before near-field microscopy will be accessible to the wide range of cell biologist. The most important improvement involves the ability to view the sample with successively overlapping resolutions and to decide which region of the sample needs to be viewed with the higher resolution that is chgaracteristic of near-field microscopy. For this investigation it is imperative to be able to build a near-field microscope in combination with a conventional light microscope. The structural constraints of near-field microscopes and the nature of biological materials and cellular problems crucially defines the characteristics of the light microscope that has to be used. In principle, these constraints require one of the many inverted light microscopes presently available. For this next generation version of the near-field microscope we have chosen the Zeiss with the ability to use in concern with the other available objectives a high numerical aperture relatively long walking distance objective that permits a large amount of the fluorescent light to be collected from the sample. The near-field microscope with the pipette is then built on top of the sample chamber and the pipette can be viewed in epi-illumination from the bottom and placed over the specific area of the sample that requires the higher resolution. This combination permits the experimenter to progress from the lower resolutions of conventional light microscopy to the highest resolutions achievable with near-field scanning optical microscopy. This combination of a near-field and a standard inverted microscope should allow for numerous biological problems to be tackled with both lens-based and lensless microscopic techniques.

Among these new emerging lensless techniques is the method of molecular exciton microscopy (MEM) in which energy transfer is used between a crystal of donor that is sitting in the tip of the pipette and an acceptor conjugated to a selective group of molecules in the cell membrane [19]. As has been argued MEM has the potential for single molecule imaging with light and thus with the combination of microscopes discussed above it should be possible to smoothly progress in one instrument from low resolution conventional light microscopy to

intermediate resolution confocal light microscopy and finally to lensless light microscopy with the potential to place in the hands of cell biologists a microscope with a variety of magnifications from a few hundred to a few million. The future for light microscopy in cell biology is exceedingly bright and a crucial aspect of this bright future is super resolution lensless light microscopy.

In summary, although these exciting developments in our laboratory have not yet yielded fruit for the ocular hazards program, we are confident, based on the above progress that in the next grant period we will get the first super-resolution light microscope images of dynamic alterations in retinal tissue with laser irradiation.

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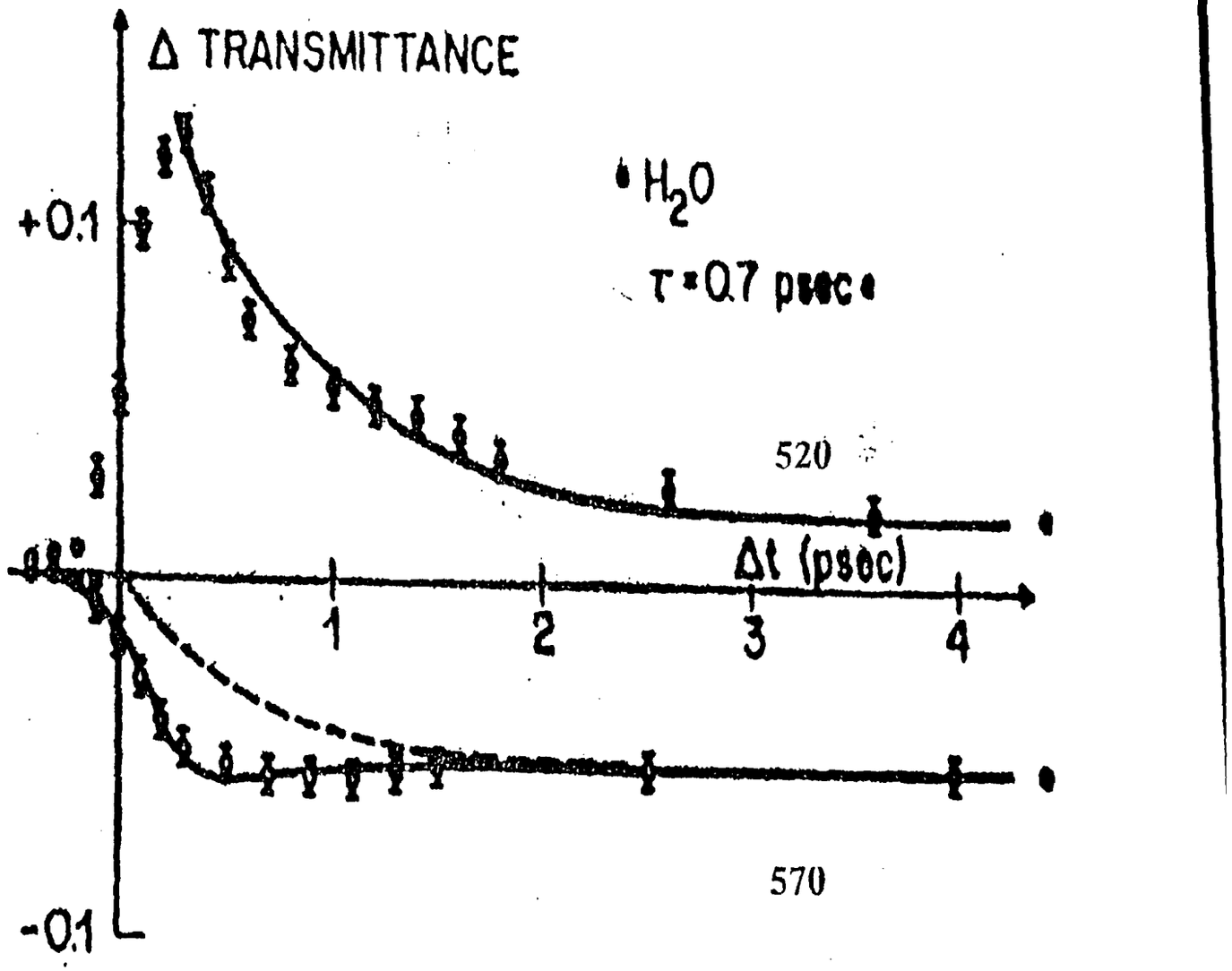


Figure 1. Absorption changes monitored at 520nm and 570nm with with 165 femtosecond pulses in native bovine rhodopsin.

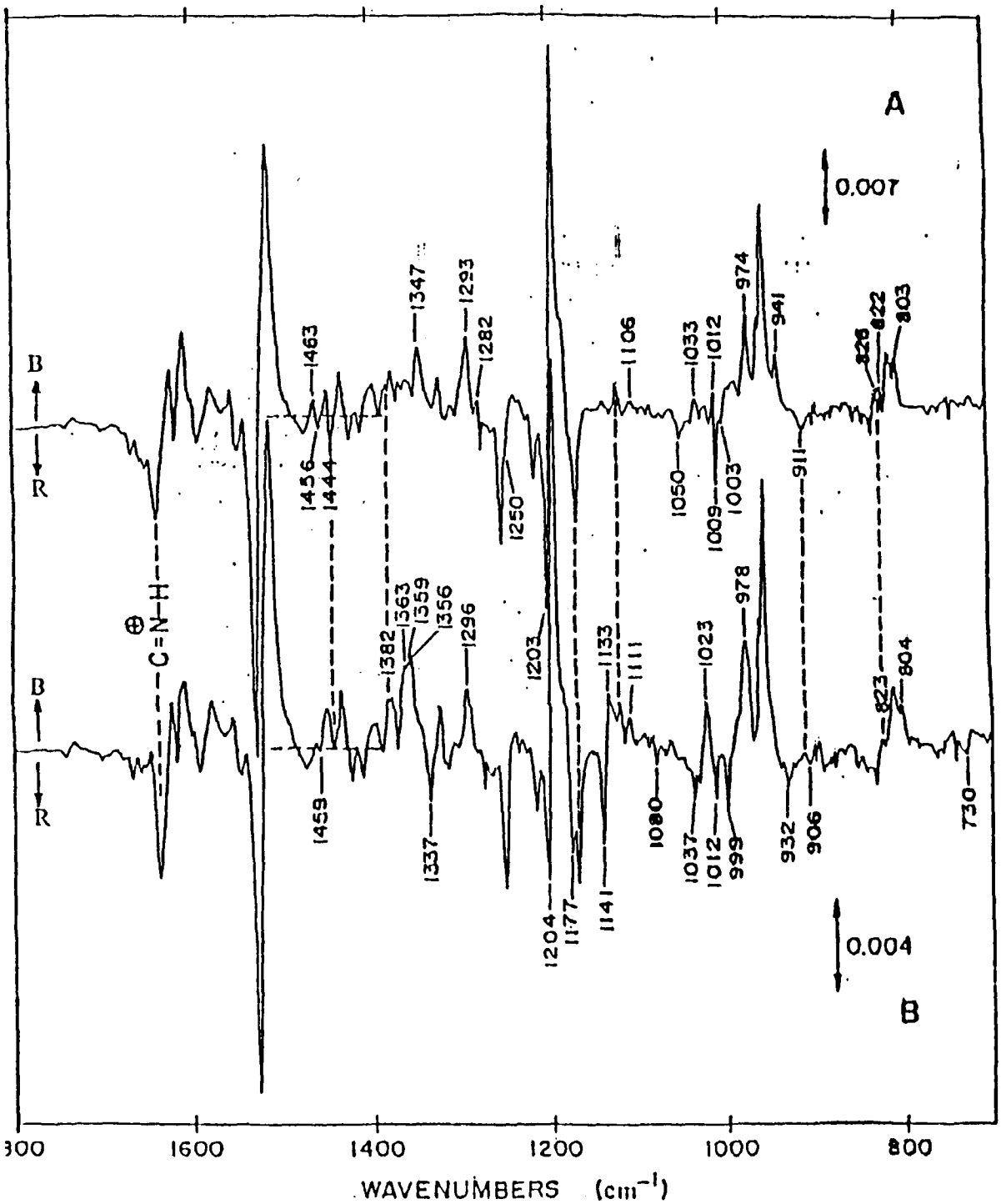


Figure 2 A. FTIR Difference spectrum between native rhodopsin (R) and the red state (B).

Figure 2 B. FTIR Difference spectrum of rhodopsin labelled with fully deuterated lysine (R) and its red absorbing intermediate (B).

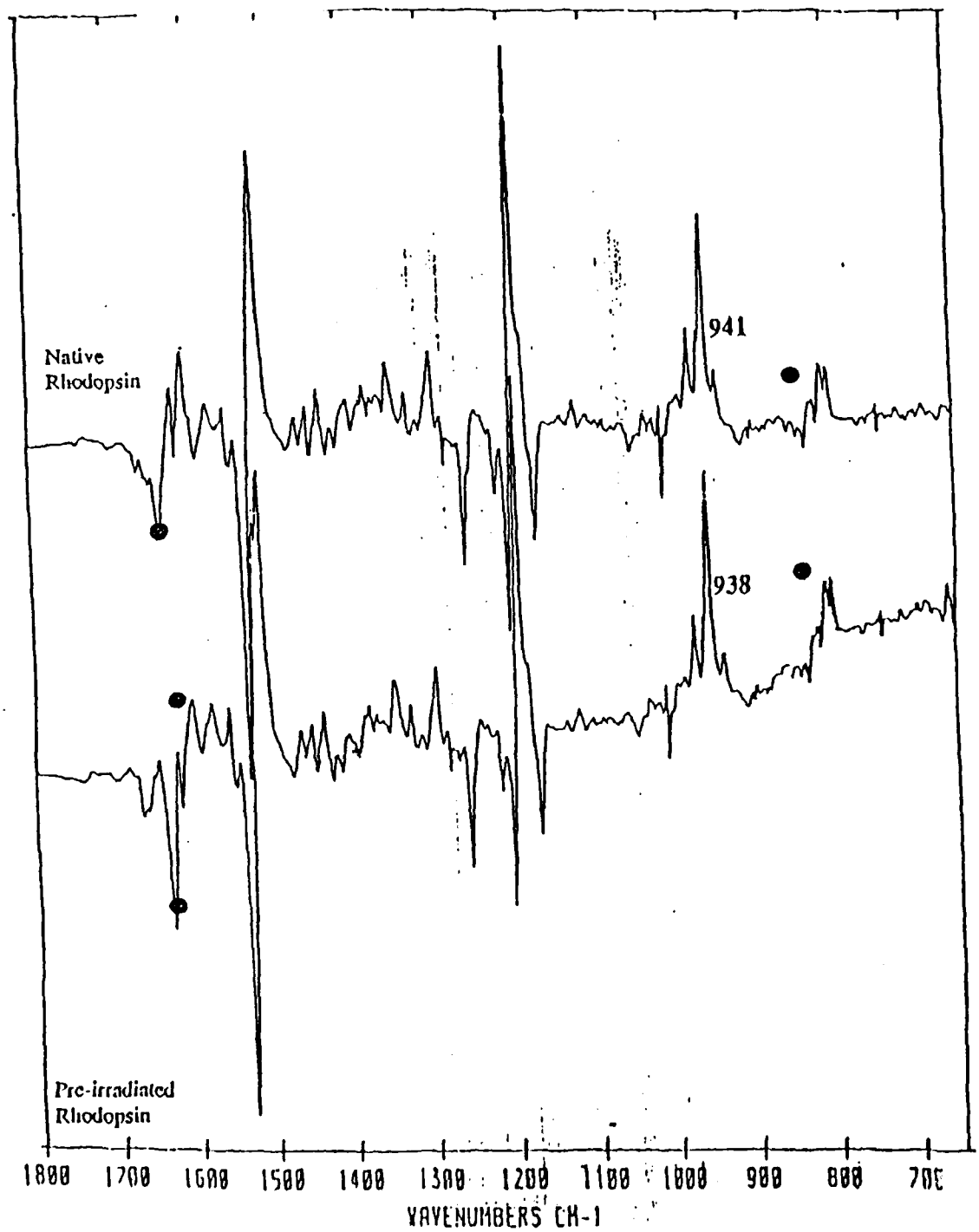


Figure 3. Comparing the FTIR Difference Spectra of native and laser pre-irradiated rhodopsin

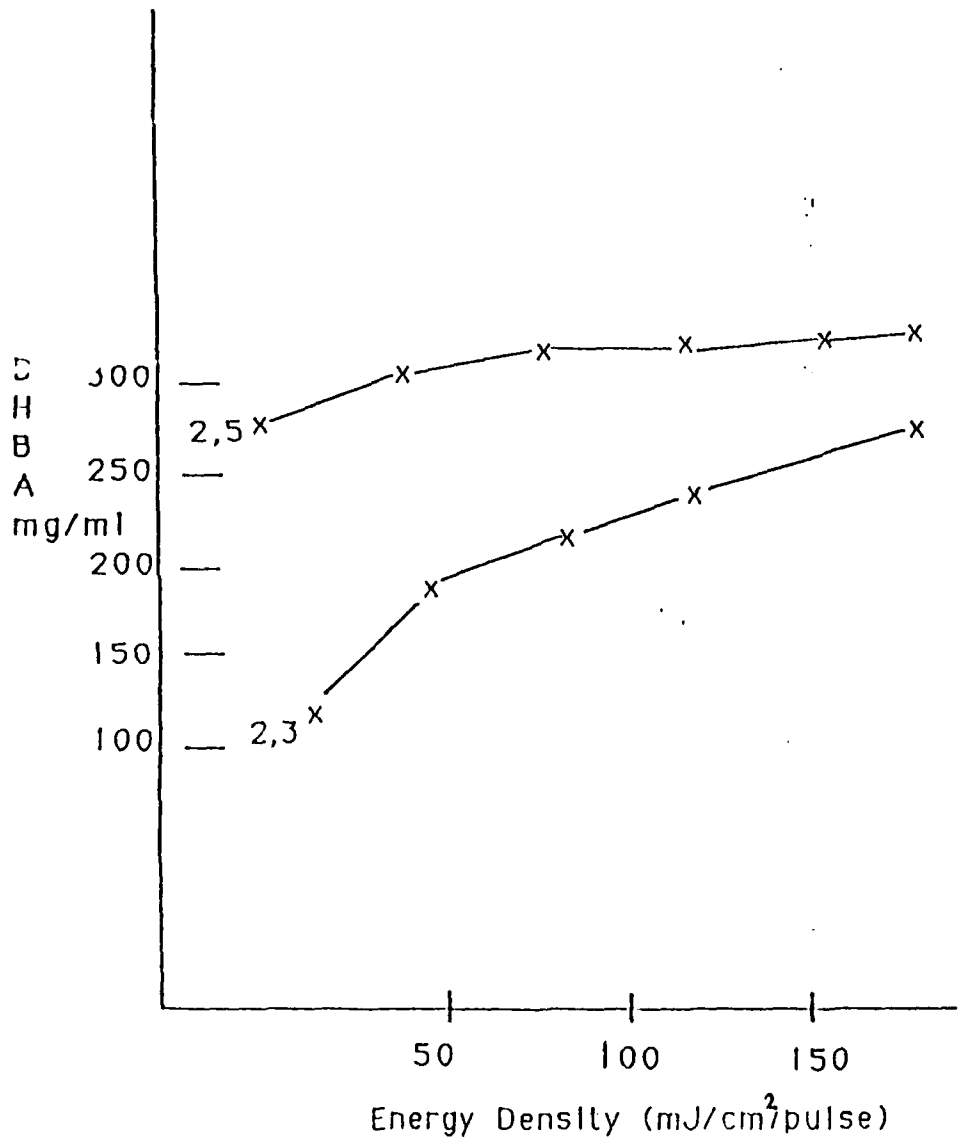


Figure 4. Changes in concentration of 2,3 and 2,5 dihydroxybenzoic acid (DHBA) as a function of the energy density of a Nd:YAG 10 nsec laser pulse.

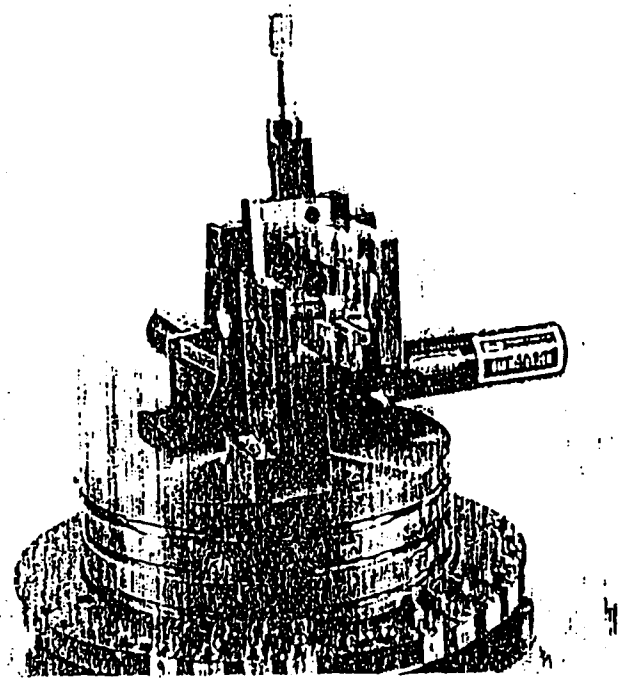
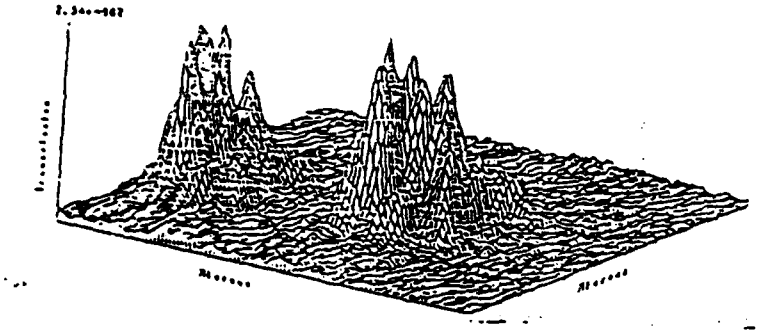
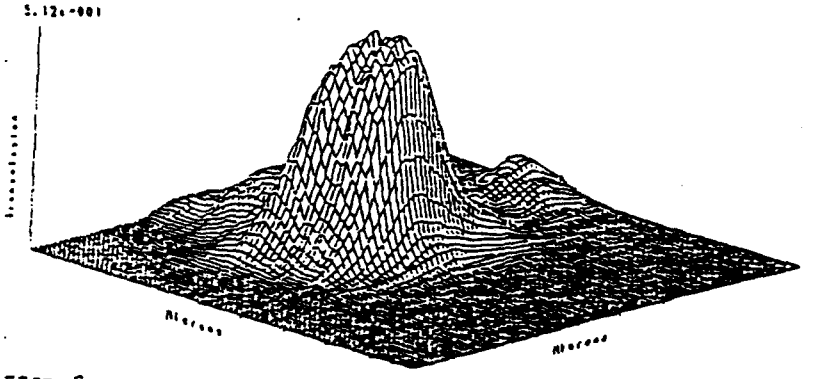


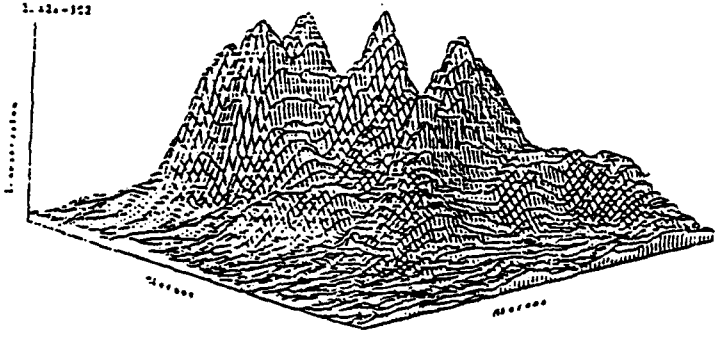
Figure 5. The Jerusalem Microscope. Vibration isolation is achieved with a stack of stainless steel plates. There is an x, y, z course stage composed of computer driven Newport translators. The pipette is held in a cylindrical piezoelectric transducer that is used for fine 0.1 Å resolution. The whole microscope is enclosed in a box for thermal and electrical isolation. The microscope works on a table top in a laboratory that includes several bays with a number of experiments simultaneoulsy in operation.



Topmost Scan



Bottom Scan



Middle Scan

Figure 6. A sequence of scans of two apertures in a metal plate illuminated by 0.1 mW of 514.5 nm light of an argon ion laser with a 0.25 μ pipette being advance closer to the apertures in the the metal plate. In the topmost scan the pipette tip is a micron away from the metal plate in which the 0.5 μ apertures are impressed. The x y scale in this image is 2 μ per large division. The middle scan has a scale of 1 μ per large division and the bottom scan has a scale of 0.1 μ per large division.